A UPLC-MS/MS Method for Quantitative Determination of Intact 5-Methyltetrahydrofolate and its Polyglutamyl Derivatives in Raw Vegetables

Chao Wang, Ken M. Riedl, Steven J. Schwartz

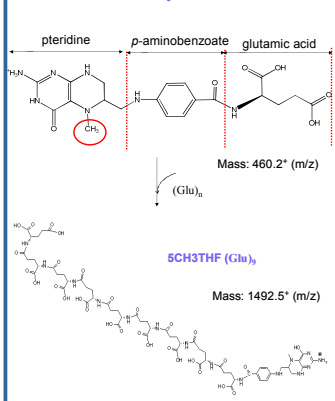
Dept Food Science & Technology, The Ohio State University

Abstract

Folate is a B-vitamin essential for human health. 5-Methyl tetrahydrofolate (5MTHF) and polyglutamatyl derivatives thereof account for 75%-100% of folate in many fruits and vegetables although reliable data about intact folate forms is lacking. Quantitatively, determination of intact folate is often limited by complicated extraction and clean-up procedures as well as limited sensitivity of detection. In addition, sample handling and workup initiates enzymatic hydrolysis to mono- and short-chain polyglutamyl derivatives. Here we report a simple and complete extraction method and ultra-high performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) for the determination of intact folate in vegetables. Samples (14 different vegetables) were steamed to inactivate enzymes and extracted in an ammonium acetate buffer containing ascorbic acid, beta-mercaptoethanol and 5MTHF stable isotope (internal standard, IS). The folate species were separated in 9min on a C18 column using a gradient of 0.1% aqueous formic acid/acetonitrile. UPLC eluate was interfaced with a triple quadrupole mass spectrometer operated in electrospray positive modeThe respective pseudomolecular cation of each 5MTHF species was selected for fragmentation to a 5MTHF fragment for detection.
We profiled folates in vegetables from *Brassicaceae*, *Asteraceae* and *Amaranthaceae* families of which most have not been profiled previously. Vegetables from *Asteraceae* and *Amaranthaceae* contained similar amounts of monoglutamyl and polyglutamyl 5MTHF species while *Brassicaceae* was dominated by polyglutamyls. The precision of the method was ≤9% RSD with 91% recovery and linearity from 0.25 nmol to 27 nmol 5MTHF. Further research will apply the method to monitor the formation of folate derivatives during food preparation and processing.

1. Introduction

$5CH_3THF$ and its derivatives consist with three parts: pteridine, p-aminobenzoate and polylglutamate chain. According to Gregory, folate-dependent enzymes prefer polyglutamate folate, so polyglutamate derivatives affect cofactor activity and transport of folates. Also the polyglutamate derivatives can protect the folate from oxidation by favoring protein binding.



2. Materials and Methods

Fresh Vegetables Analyzed

All vegetables purchased from Whole Foods except Endive and Turnip Greens (Meijer).

Standards and Stable Isotope Internal Standard

$5CH_3THF$, $5CH_3THF(Glu)_2$, $5CH3THF(Glu)_3$, $5CH3THF(Glu)_4$ and $5CH3THF(Glu)_5$, are all sodium salts. Stable **Isotope Internal Standard:** $5CH_3$ [$^{13}C_5$] Glu, calcium salt

Sample Preparation

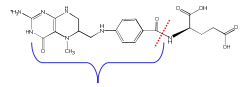
- Steam 5g vegetables (fresh weight) suspended above 1L of boiling water for 10min;
- Grind steamed vegetables in liquid nitrogen with mortar and pestle
- Add 20mL boiling extraction buffer (100mM ammonium acetate, 1% ascorbic acid, 0.2% beta-mercaptoethanol and internal standard, pH=7.9), return to boiling for 10min
- Cool to room temperature, vacuum filter using Whatman #1 and bring to 25mL
- Filter supernatant with 0.22μm Nylon filter for UPLC injection

UPLC Parameters

Column: Sunfire C18 (4.6×150mm I.D.; 5μm); Column Temperature: 40° C ; Mobile Phase: (A) aqueous 0.1% formic acid and (B) acetonitrile ; Gradient Elution: 0-4min, 0-20%B; 4-5min, 20-95%B; 5-6.5min, 95%B; 6.5-9min, re-equilibrate. Injection Volume: 50μl; Flow Rate: 1.8mL/min

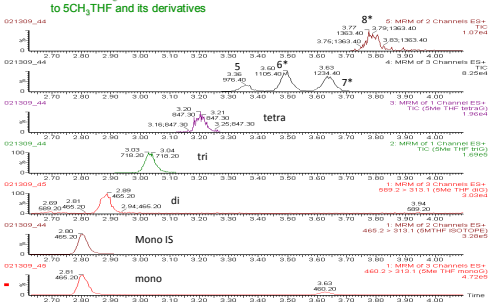
MS/MS Parameters

Capillary Voltage: 3.2kV; Source Temperature: 110° C; Desolvation Temperature: 400° C; Cone Gas Flow: 110L/hr , Desolvation Gas Flow: 800L/hr; Cone Voltage and Collision Energy: optimized for each compound; RF Lens 1: $5CH_3THF$, $5CH_3THF(Glu)_2$ and $5CH_3$ [$^{13}C_5$] Glu 10V, other compounds 40V



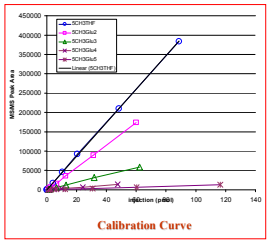
313+ m/z fragment common to $5CH_3THF$ and its derivatives

***authentic standards unavailable but quantify in equivalents of 5CH3THF penta-glutamyl. Parent>daughter transition and elution order consistent with species.**



Characterizing the Quantitative Method

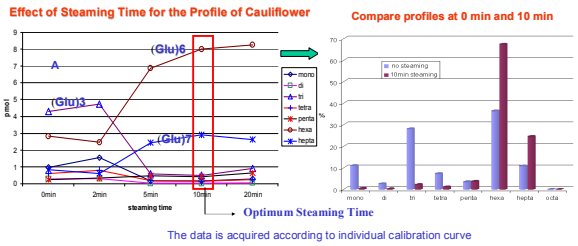
Compound	LOD (fmol)	LOQ (fmol)	Slope (peak area/pmol)	R^2	Linear range (injection pmol)
5MTHFGlu	156	472	4333	0.9999	0.3-10.8
$5MTHFGlu_2$	64	193	2886	0.9993	1.2-60
$5MTHFGlu_3$	301	911	943	0.9989	1.2-62.3
$5MTHFGlu_4$	261	792	286	0.9996	0.9-47.5
$5MTHFGlu_5$	425	1290	115	0.9991	1.2-116.2
$5MTHFGlu_{6-9}$*	425	1290	115	0.9991	1.2-116.2



*$5MTHFGlu_{6-9}$ – since authentic standards were unavailable the response was determined using the response of the $5MTHFGlu_5$

3. Results & Discussion

Optimized Steaming to Inactivate Gamma-glutamylase



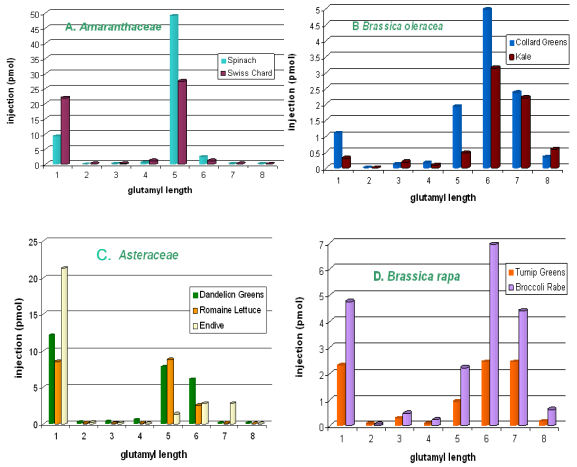
The data is acquired according to individual calibration curve

A shows $5CH_3THF$hexaglutamate and heptaglutamate is inhibited to convert to mono-, di- and triglutamate with increasing steaming time. It is ascribed to the steaming inactivate gamma-glutamylase progressively

B. Comparing no steaming to 10 min steaming, the percentage of triglutamyl decreases 25.8%, monoglutamyl decreases 9.4%, tetraglutamyl decreases 6.2% and diglutamyl decreases 1.4%. The total percentage of monoglutamyl-tetraglutamyl decreases 43%. While the percentage of hexaglutamyl increases 31%, heptaglutamyl increases 13%. The total percentage of hexaglutamyl-heptaglutamyl increases 44%.

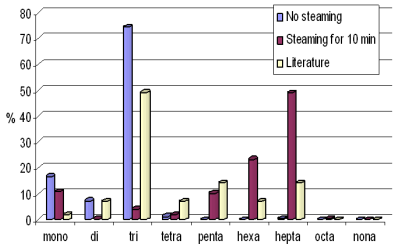
Hexaglutamyl-heptaglutamyl prefer to convert to triglutamyl.

Polyglutamyl $5CH_3$ THF Profiles of Same Family or Species of Representative Vegetables



A-D. shows the $5CH_3THF$ and its derivatives distribution of same family or same species are similar. And different family has unique profile. Vegetables from *Asteraceae* and *Amaranthaceae* contained similar amounts monoglutamyl and polyglutamyl 5MTHF species while *Brassicaceae* was dominated by polyglutamyls.

Polyglutamyl $5CH_3THF$ Profiles Compared to Literature for Broccoli



The profile of literature (Verlinde, P., *Food Chemistry*, 11(2008):220-229) shows similarity with our profile without steaming.

4. Conclusions

1. The extraction method showed progressively inactivate endogenous enzyme, maximum extracting efficiency and linearity for profiling intact $5CH_3THF$ and its derivatives. This is a big improvement compared to the existing method.
2. $5CH_3THF$and its derivatives distribution of the same family or species are similar. Vegetables from *Asteraceae* and *Amaranthaceae* contained similar amounts monoglutamyl and polyglutamyl $5CH_3THF$ species while *Brassicaceae* was dominated by polyglutamyls
3. Data from this report adds to the USDA database for Swiss chard, bok choy and broccoli rabe.

Reference and Acknowledgement

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